# The emerging role of DNA methylation in kidney transplantation: a perspective

Line Heylen, M.D.[1,2,3,4]; Bernard Thienpont, Ph.D.[3,4]; Maarten Naesens, M.D., Ph.D.[1,2]; Diether Lambrechts, Ph.D.[3,4]; Ben Sprangers, M.D., Ph.D.[1,2].

[1]Department of Nephrology and Renal Transplantation, University Hospitals Leuven, Leuven, Belgium

[2]Department of Immunology and Microbiology, KU Leuven, Leuven, Belgium.

[3]Laboratory of Translational Genetics, Department of Oncology, KU Leuven, Leuven, Belgium

[4]Vesalius Research Center, VIB, Leuven, Belgium

**Corresponding author:** Line Heylen: line.heylen@med.kuleuven.be

**Running title:** DNA methylation research in kidney transplantation

**Key words:** kidney transplantation, epigenetics, DNA methylation, senescence, ischemia, fibrosis, immune response

**Abbreviations:**

5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; CKD, chronic kidney disease; DGF, delayed graft function; DNMT, DNA methyltransferase; ESRD, end-stage renal disease; SNP, single nucleotide polymorphism; TDG, thymine DNA glycosylase; TET, ten-eleven translocation methylcytosine dioxygenase enzyme; Th cell, T-helper cell; Treg, regulatory T-cell; TTS, transcription start site

## Abstract

Allograft outcome depends on a range of factors, including donor age, the allo-immune response, ischemia-reperfusion injury, and interstitial fibrosis of the allograft. Changes in the epigenome, and in DNA methylation in particular, have been implicated in each of these processes, either in the kidney or other organ systems. This review provides a primer for DNA methylation analyses and a discussion on strengths and weaknesses of current studies, but also a perspective for future DNA methylation research in kidney transplantation. We present exciting prospects for leveraging DNA methylation analyses as a tool in kidney biology research, and as a diagnostic or prognostic marker for predicting allograft quality and success. Topics discussed include DNA methylation changes in aging, and in response to hypoxia and oxidative stress upon ischemia-reperfusion injury. Moreover, emerging evidence suggests that DNA methylation contributes to organ fibrosis and that systemic DNA methylation alterations correlate with the rate of kidney function decline in patients with chronic kidney disease and end-stage renal failure. Monitoring or targeting the epigenome could therefore reveal novel therapeutic approaches in transplantation and open up paths to biomarker discovery and targeted therapy.

**Main Body Text**

Epigenetics involves the potentially stable and, ideally, inheritable mechanisms that control gene expression and that occur without changes in the DNA sequence(1). They can be generally grouped into three main categories: methylation of DNA, modifications of histones and changes in the positioning of nucleosomes(2). The interactions between these different epigenetic modifications determine their effect on gene expression and are fundamental to the regulation of many cellular processes. Epigenetic changes are often triggered by developmental, environmental or pathogenic stimuli and, because of their stability and heritability, can produce long-lasting cellular phenotypes(2). As such, they provide an interface between the environment and gene expression(1). In recent years, we have witnessed a growing interest in epigenetic research in various research domains, such as cancer or auto-immunity. Also in the field of nephrology, interest has steadily been growing. However, so far, only few studies have focused on the role of epigenetic changes in kidney transplantation. This is somewhat surprising, as many key factors that influence DNA methylation, which is the best characterized epigenetic mark, are of major prognostic significance in kidney transplantation. Indeed, advanced donor age, alloreactive immune responses, ischemia-reperfusion injury and fibrosis significantly contribute to allograft survival and at the same time these factors also trigger DNA methylation changes.

Therefore, this review aims to provide a perspective on the emerging role of DNA methylation in kidney transplantation. By pointing out major discoveries in related

research fields, we aim to highlight how epigenetic research can provide novel insights into the mechanisms of kidney transplant failure, and may thus contribute to improving long-term allograft survival.

## An introduction to DNA methylation and demethylation

DNA methylation occurs almost exclusively at cytosines located in the context of a CpG dinucleotide(3). Although CpG dinucleotides are rare in the genome, a small fraction is clustered into so-called “CpG islands”. CpG islands have a high CpG density, are unmethylated under normal conditions and mostly co-localize with promoter regions in the genome(4, 5)(Figure 1). Methylation of CpG islands in promoters represses gene expression, by displacing transcription factors and attracting methyl-binding proteins that trigger gene silencing(6). DNA methylation leading to gene silencing is essential for dosage compensation in chromosome X-inactivation, for parent-of-origin-dependent imprinting, and more generally for cell differentiation and mammalian development(2) and has a major role in long-term gene expression silencing. In the CpG context, with methylation on both DNA strands mirroring each other, DNA methylation patterns can be faithfully copied upon DNA replication, and transmitted to both daughter cells. As a consequence, DNA methylation patterns are stable, unless when they are actively altered. Such alterations occur extensively during development, and DNA methylation patterns are therefore cell-type and lineage specific. It thus endows unique functions to different cells and tissues that have identical

genomes(7-9). For instance, the DNA methylome of the kidney closely resembles the DNA methylome of other mesoderm-derived tissues(7). In addition, genes encoding kidney-specific transporters are hypomethylated in the rodent kidney compared to other organs(10). When specifically focusing on rodent proximal tubular cells, again genes that were hypomethylated compared to the kidney in general included those that were essential for proximal tubular cellular function(11).

DNA methylation is maintained during cell division by the action of DNA methyltransferase 1 (DNMT1)(2). Recruted together with other components of the DNA replication machinery during the S-phase, DNMT1 binds to hemimethylated CpG sites and methylates the CpG on the newly synthesized unmethylated daughter strand, thus propagating methylation patterns and ensuring the stability of the epigenetic code. On the other hand, *de novo* methylation is orchestrated predominantly by the action of DNMT3a and DNMT3b(2). These methyltransferases are capable of methylating unmodified CpG sites. *De novo* methylation primarily occurs during embryogenesis at the majority of CpG sites in the genome to establish the basal pattern of DNA methylation, and subsequently targeted during early development to repress genes involved in pluripotency(12). Following differentiation, cells lose their de novo methylation activity and their DNA methylome is further maintained during cell replication through DNMT1 activity. Still, DNMT3a and DNMT3b are

ubiquitously expressed at low levels in adult tissues, and certain circumstances can trigger de novo methylation of genes(12).

The reverse process, DNA demethylation, was initially thought to occur passively. Recent evidence however identified active demethylation through the ten-eleven translocation methylcytosine dioxygenases (TET1, TET2 or TET3). These enzymes oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC)(13), leading to DNA demethylation(14)(Figure 1). Global erasure of the methylome occurs at specific stages of mammalian development(15). However, TET proteins are also ubiquitously expressed at varying degrees in non-dividing somatic cells, and active DNA demethylation can occur in most cell types (3).

Some studies suggest that 5hmC is more than an intermediate to demethylation, having the potential to act as a *bona fide* epigenetic mark in its own right(16). In highly expressed genes, 5hmC levels are low around the transcription start site and increase towards the 3' end of the gene(16). Moreover, hydroxymethylation is a stable DNA modification and tissues with differentiated, non-proliferating cell types have a higher 5hmC content(17). This is particularly true for the brain, but also in the kidney 5hmC levels are relatively abundant(17).

A comprehensive picture of the regulation of DNA methylation turnover is currently lacking(3). Still, it is evident that it is the interaction between different

epigenetic players that ultimately determines gene expression. For example, the effect of DNMTs on DNA methylation is influenced by their interaction with histones and nucleosomes, while on the other hand DNA methylation can also mediate histone and nucleosome modifications(2). In addition, genetic variability is an important determinant of the DNA methylation profile, probably mediated through variability in transcription factor binding, another factor influencing DNA methylation(3). Finally, also environmental stimuli can influence DNA methylation(1). In this review, we will additionally focus on factors that are relevant for transplantation research.

We will now discuss how the major prognostic factors determining survival of kidney transplants are known to have a profound impact on DNA methylation. As such, there might be unanticipated roles for DNA methylation in determining the long-term outcome.

**DNA methylation and cellular aging**

Advanced donor age is one of the key variables determining worse long-term outcome of kidney transplantation(18). Several underlying mechanisms have been proposed, including telomere shortening, accumulation of somatic mutations and oxidative stress, although their exact causality remains unclear(19). Only recently, epigenetic changes accumulating during cellular senescence have been detected. Indeed, with advanced age the variability in DNA methylation patterns increases significantly, a process referred to as

epigenetic drift(20). This increase in interindividual variability with advancing age is accompanied by an increase in methylation at sites with low methylation levels, such as CpG islands in promoters, and a decrease at sites with high methylation levels, such as nonisland CpGs(21).

Interestingly, the link between age and DNA methylation is robust and does not seem to depend on the micro-environment, as one year after allogeneic hematopoietic stem cell transplantation, DNA methylation in peripheral blood cells, which originate from the donor stem cells, still correlates with donor age but not with recipient age(22). It has furthermore been suggested that aging affects the epigenome in a tissue-specific manner. For instance, the promoter of N-cadherin, a cell adhesion molecule essential for cell polarity and thus renal function, is strongly methylated in aged, but not young, murine kidneys, which explains the age-dependent decrease in renal N-cadherin expression. Neither young or old rat livers display such a change in methylation or gene expression profile(23).

Besides the increased variability in DNA methylation with age, the methylation state at certain CpGs are highly correlated with age, to the extent that they can be used to accurately predict chronological age(21). This process is referred to as the epigenetic clock. In this respect, a multi-tissue predictor of age was recently built by comprehensive analysis of publicly available DNA methylation data of 8000 samples encompassing 51 healthy tissues and cell types, including the kidney. At 353 CpGs across tissues, the methylation state predicted age(24). This illustrates that DNA methylation changes capture biological age, similar to

telomere shortening. In addition, the deviations of the age estimated by DNA methylation analysis from the chronological age independently associated with increased mortality, suggesting that methylation-based age can provide an better estimate of future lifespan than chronological age(25). Unfortunately, very little is known about age-related changes in the DNA methylome of the kidney. Moreover, the function of these epigenetic changes with age and the underlying mechanisms driving them are currently unknown(21). The role of epigenetic dysregulation in allografts from older donors thus warrants additional investigation.

**DNA methylation and the immune response**

Allo-immune responses to the allograft impede successful kidney transplantation, and are characterized by the differentiation of progenitor and intermediate cell types to fully differentiated immune cells via a tightly regulated cascade of gene expression changes. Recent evidence supports a role for DNA methylation changes in orchestrating this process(Figure3).

Indeed, early on in hematopoiesis, hematopoietic stem cells differentiating into the myeloerythroid versus lymphoid lineage achieve markedly different methylation patterns(26, 27). In addition, active demethylation by TET2-mediated hydroxymethylation is involved in the differentiation of peripheral blood monocytes into macrophages and dendritic cells (28-30). Also in the activation of macrophages towards the two distinct polarized M1 (representing the ‘classical’ activation of macrophages, associated with pro-inflammatory responses) and M2

(representing the ‘alternative’ activation, associated with anti-inflammatory responses and tissue remodeling) phenotypes, differential DNA methylation is involved. Inhibition of DNMT3B is namely able to skew macrophages towards the M2 phenotype(31). Interestingly, M2 macrophages are involved in renal regeneration by generating mediators that support tubular survival and proliferation to restore tubular integrity (32-34). This epigenetic regulation of macrophage polarization thus supports the idea of a continuum of macrophage phenotypes, rather than fixed polarized phenotypes(35).

Epigenetics are also involved in the differentiation of naïve CD4+ T cells toward effector T helper cells (Th1, Th2, and Th17) or regulatory T (Treg) cells(36). For example, the segregation of the Th1 and Th2 cells based on their mutually exclusive production of interferon–γ (IFNγ) or interleukin-4 (IL-4), IL-5 and IL-13, respectively, is characterized by their corresponding different DNA methylation profiles (37). Also for Treg cells that constitutively express Foxp3, a transcription factor necessary for its suppressive function, maintaining a stable expression of Foxp3 relies upon DNA demethylation of a CpG-rich region within the Foxp3 locus early in thymic Treg development(38-40). In B cells that are activated in the germinal center by antigenic stimulation, global reprogramming of the DNA methylome occurs(41). Thereafter, when differentiating into memory B cells or plasma cells, the DNA methylome remains mostly unaltered, providing a platform for memory B cells to rapidly differentiate into plasma cells upon antigen rechallenge(41) .

As epigenetic mechanisms determine thus both lineage stability as well as lineage plasticity, targeting the epigenome could be promising as a therapeutic strategy to prolong kidney transplantation, especially in the era of cell therapy in transplantation medicine. For example, Treg cell therapy might improve allograft survival but is hampered by the loss of FOXP3 expression during cell expansion and transfusion. The inhibition of DNA methylation by DNMT inhibitors could potentially promote more stable FOXP3 expression in Tregs(42). In addition, epigenetic therapies could be applied to skew macrophages towards the regenerative, anti-inflammatory M2 phenotype, and induce less deleterious rejection phenotypes(31). The compelling aspects of therapy targeting the epigenome are discussed in the last section.

Furthermore, there is an unmet need for monitoring the immune response in transplant recipients as sufficiently robust biomarkers to predict long-term outcome are currently lacking. In oncology, aberrant DNA methylation patterns are used for cancer detection, prognosis and prediction of therapeutic responses(43). Only few studies evaluated the role of DNA methylation in transplantation medicine. In kidney transplant recipients with subclinical rejection, long-term allograft outcome was better when FOXP3+ Treg cells were present in allograft biopsies. In these FOXP3+ Treg cells, a locus near *FOXP3* was unmethylated which distinguishes them from effector T cells, thus serving as a protective biomarker(44). Likewise, in hematopoietic stem cell transplantation, DNA methylation at genes encoding IFNγ, FASL and IL-10 was associated with

the severity of graft-versus-host disease(45). DNA methylation markers can thus be used to assess the renal cell composition, and monitor the immune response in transplanted patients. Moreover, DNA methylation entails advantages over proteins and RNA as a biomarker of immune activation by being more accessible, which is discussed in the last section of this minireview.

Finally, besides controlling immune cell differentiation and activation, epigenetic regulators are themselves susceptible to inflammation and immune responses. For instance, IL-6 is able to elicit epigenetic changes by regulating DNMT expression(46). Moreover, several associations between chronic inflammation and epigenetic alterations have been observed(47). Although these studies suggest an impact of inflammation on epigenetics, much more work needs to be done to understand the relevance of this link.

**DNA methylation and ischemia-reperfusion injury**

Another factor that adversely impacts kidney transplantation outcome is ischemia-reperfusion injury occurring during transplantation. Indeed, prolonged cold ischemia is associated with increased risk of delayed graft function (DGF)(48), diminished allograft function(49), chronic allograft injury(50) and worse allograft survival(51).

Ischemia and oxidative stress are thought to affect DNA methylation, and several underlying mechanisms have been proposed. First, oxidative stress can alter the DNA structure, by causing base modifications, deletions, strand breakage,

chromosomal rearrangements, and other genetic changes(52). These DNA lesions could influence the binding of DNMTs and methyl-binding proteins, thereby affecting DNA methylation and influencing transcription(52). Another example of oxidative DNA damage is the formation of 8-hydroxy-2-deoxyguanosine (8-OHdG), whose presence strongly inhibits methylation of adjacent cytosines(52). Second, TET enzymes responsible for active demethylation use molecular oxygen as co-substrate, suggesting that DNA demethylation is arrested during ischemia-reperfusion and gene expression is changed accordingly(13). In addition, oxidative stress also affects alpha-ketoglutarate and Fe(II) levels, which similar to oxygen serve as co-factors for TET-mediation demethylation(53, 54). Finally, hypoxia can also directly modulate the expression of DNMTs and TET enzymes(55-58). For instance, DNA binding consensus sequences for hypoxia-induced transcription factors have been identified in the DNMT1 and DNMT3b promoters(57). Taken together, there are numerous mechanisms through which ischemia and oxidative stress could impact DNA methylation, but a conclusive picture is currently lacking.

The theoretical association between hypoxia and DNA methylation has been explored in different tissues and animal models, albeit only to a very superficial extent. In prostate cells, neurons, cardiac and pulmonary fibroblasts, hypoxia or ischemia both increase DNA methylation as measured by immunostaining or by [$^{3}$H]methyl incorporation(56, 57, 59, 60). Other small studies examined ischemia-reperfusion injury in the rodent kidney. One observed reduced 5hmC levels with unaltered 5mC levels by immunodot blotting(55). Another focused at only one

CpG in the C3 gene promoter, as renal C3 synthesis increases after ischemia-reperfusion injury and contributes to the injury. At this locus, demethylation was observed after ischemia, with further demethylation after reperfusion(61). In a syngeneic rat transplantation model, kidneys characterized by more severe chronic injury similarly displayed aberrant methylation of the C3 locus(62). Importantly, none of the abovementioned studies investigated DNA methylation changes using a genome-wide approach, limiting the breath of conclusions that can be drawn. Moreover, except for one(60), none of them directly linked DNA methylation changes to gene expression changes.

Thus although the abovementioned studies are of interest and point towards the potential importance of epigenetic alteration in ischemia-reperfusion injury, no definite conclusions can be drawn from these small, and sometimes conflicting, studies. Additional systematic studies are needed to better define the impact of ischemia and reperfusion on kidney DNA methylation levels.

**DNA methylation and fibrosis**

Similar to wound repair, fibrosis is a process triggered by a specific injury and characterized by the deposition of extracellular matrix through activated fibroblasts. However, in contrast to wound repair, fibrogenesis can progress even after the initiating insult has disappeared(63). DNA methylation could determine why different organs respond differently to injury, either by triggering tissue repair (cfr. wound healing) or by ongoing scarring (cfr. fibrosis). In nephrology, this is of

particular interest: chronic kidney disease (CKD) is characterized by progression to tubulointerstitial fibrosis, irrespective of the initiating trigger. However, how the kidney progresses from the initial injury to end-stage disease is incompletely understood.

Epigenetic changes contribute to fibrosis in several settings, including the heart, lungs, liver and skin (64-66). For instance, epigenetic alterations could underlie cardiac fibrosis after myocardial infarction, as cardiac fibroblasts exposed to hypoxia obtain a pro-fibrotic state that is associated with global DNA hypermethylation(57). Administration of a DNMT inhibitor (5-azacytidine) moreover reduced cardiac fibrosis and improved systolic and diastolic function in a rat model of myocardial infarction(67). In nephrology, epigenetic changes have similarly been implicated in fibrosis. In fibroblasts isolated from human fibrotic kidneys, hypermethylation of *RASAL1* was detected by genome-wide DNA methylation profiling(68). RASAL1 inactivates Ras proteins, which induce proliferation. Hypermethylation-induced repression of RASAL1 can thus explain why fibroblasts are characterized by continuous proliferation. These data were validated in murine models of kidney fibrosis, and inhibition of RASAL1 methylation by 5-azacytidine moreover suppressed kidney fibrosis(68, 69). In addition, RASAL1 knockdown lowered RASAL1 expression in nonfibrotic fibroblasts to the level of fibrotic fibroblasts, phenocopying the proliferative activity and fibrotic potential as observed in fibrotic fibroblasts, suggesting that the changes in RASAL1 expression induced by increased methylation are

sufficient to promote fibrosis. Moreover, reversal of kidney fibrosis by administration of bone morphogenic protein 7 was accompanied by normalization of RASAL1 promoter methylation and by *de novo* RASAL1 hydroxymethylation, through increased TET3 expression(69). Finally, further strengthening the relevance of these findings, reduced TET3 gene expression was observed in publicly available transcriptome data of various kidney diseases(69).

Taken together, these observations reveal a critical role for DNA methylation in the pathogenesis of several fibrotic diseases including CKD. In kidney transplantation, chronic dysfunction of the allograft and graft failure are often characterized by interstitial fibrosis, but DNA methylation changes have hitherto not been investigated as a trigger of this process.

**DNA methylation and kidney function**

The abovementioned studies suggest that DNA methylation changes can be found in patients with CKD. Indeed, site-specific DNA methylation changes have already been detected in patients with diabetic nephropathy(70), in patients on chronic hemodialysis(71) and in patients with CKD in general(72). Moreover, DNA methylation changes have been associated with the rate of kidney function decline(73). However, others failed to observe an association between global DNA methylation and estimated glomerular filtration rate in patients with CKD(74). Simple evaluation of global hypo- or hypermethylation can, however,

not exclude the possibility that site-specific DNA methylation changes exist. Moreover, except for one(72), these studies focused on DNA methylation in peripheral blood instead of focusing on the renal tissue itself, thereby limiting the extent of conclusions that can be drawn from these data to merely correlative. Indeed, it is unclear whether epigenetic changes in CKD directly cause end-stage renal disease (ESRD)(72), whether they reflect environmental factors that alter DNA methylation in all tissues(75) or whether they are the result of the disease on itself(71, 76).

In line with the latter possibility, it has been demonstrated that uremic toxins, which are increased in patients with ESRD and related to the increased cardiovascular risk, can induce hypermethylation of *Klotho in vitro* in human renal tubular cells, and *in vivo* in mice(77). *Klotho* is a central player in ESRD, with *Klotho* expression being reduced in patients with ESRD, and overexpression of *Klotho* rescuing ESRD in mice. Similarly, the *Klotho* promoter is hypermethylated in renal tissue and in peripheral blood mononuclear cells of patients with CKD, with the degree of hypermethylation correlating with the clinical and histological severity of CKD(78).

**Challenges in epigenetic research and look at the future**

Epigenetics is a novel and booming research field. Many questions unanswered today will be tackled tomorrow. When its characteristics become unraveled, the future of translational epigenetic research will become evident. Until then, we can only speculate that the unique features of epigenetics hold promise for

pathogenic insight, biomarker discovery and targeted therapeutics also in transplantation.

The attractiveness of DNA methylation as biomarker lies in its feasibility. It is less sensitive to tissue handling compared to RNA or proteins and can even be performed on DNA isolated from small amounts of fixed tissue(3). Moreover, given the recent interest in donor-derived cell-free DNA as a biomarker in transplantation(79), its DNA methylation profile merits further investigation. As for every biomarker, a mechanistic understanding of the underlying biology is not strictly required, as its sole purpose is to provide a diagnostic or prognostic handle.

On the other hand, when performing epigenetic research to better understand the processes associated with transplant failure, compelling aspects of epigenetics need to be addressed. First, the region in which DNA methylation changes take place determines the effect on gene function, and given the complexity of transcription regulation, straightforward effects are not always to be anticipated. Therefore, DNA methylation research should take the site-specificity and the transcriptional response into account.

Second, epigenetic research is complicated by cell- and tissue-specific methylation patterns. This is particularly relevant when performing DNA methylation analyses on tissue biopsies(3). When, for example, one would compare DNA methylation of allograft biopsies with acute cellular rejection to allograft biopsies without rejection, the presence of inflammatory cells could

heavily influence the results. This underlines also why investigating the primary organ is superior to studying more accessible secondary derivatives such as peripheral blood.

Third, although cell lineage is the major determinant of DNA methylation patterns, they can also be influenced by genetic differences(3, 80). Inter-individual variation needs thus to be considered and sites with consistent levels of methylation in healthy individuals need to be identified before DNA methylation profiling can find its way to clinical applications(81). Current efforts to develop reference DNA methylation data sets are therefore of utmost importance(81). Also genetic variability between individuals can lead to the erroneous identification of differentially methylated regions, as single nucleotide polymorphisms (SNPs) can disrupt CpG dinucleotides(80) directly influencing their methylation. In addition, bisulfite treatment of DNA, necessary for DNA methylation analysis, converts unmethylated cytosines to thymines, while methylated cytosines are read as cytosines. Therefore, a C to T mismatch SNP can be falsely recognized as an unmethylated cytosine(80).

Fourth, besides gender, age and inter-individual differences, other factors relevant for the transplantation setting could change DNA methylation, and thus confound epigenetic research results. For example, HTK preservation solution contains alpha-ketoglutarate, which is an important cofactor for TET activity. DNA methylation changes following the use of this preservation solution have not been investigated yet. In addition, the epigenetic effects of immunosuppressive drugs,

such as corticosteroids(82), need to be established. Moreover, there is currently insufficient knowledge on the numerous other factors that could modify the DNA methylome, such as nutritional deficiency, inflammation, smoking and obesity.

Fifth, future studies should entail an unbiased approach by investigating the DNA methylome in a genome-wide manner to provide a broader as well as a more in-depth analysis than when only a specific set of genes is studied. Current methylation studies moreover fail to discriminate cytosine methylation from hydroxymethylation. The development of more specific assays is anticipated to provide an even greater sensitivity in detecting relevant epigenetic changes.

Investigating the role of DNA methylation on allograft outcome could not only aid in our understanding of why some kidney transplants fail, and therefore contribute to improved diagnostics and risk prediction. Also with respect to therapeutics, epigenetic research is promising. DNA methylation is modifiable and agents that act on these mechanisms have been developed. Still, finding dysregulated DNA methylation in rejection or chronic allograft injury does not necessarily imply a therapeutic potential of these agents equally to recent advances in haemato-oncology. For example, DNMT inhibitors like 5-azanucleosides inhibit all DNA methyltransferases. In cancer, the advantages could overwhelm its toxicity, but in transplant recipients this toxicity would likely be unacceptable. However, with recent developments in (epi-)genome engineering technologies, therapy targeted to localized DNA methylation changes will no longer be an unattainable dream(83).

By addressing these challenges, and by being receptive to discoveries in other research fields, epigenetics holds great promise for research in aging-associated, allo-immune and ischemia-reperfusion injuries in organ transplantation. Continuing this research is crucial, as we still do not succeed in improving long-term transplant outcome due to incomplete insight in the mechanisms underlying and the factors predicting allograft outcome.

In linking environmental influences to long-lasting cellular phenotypes, epigenetic changes underlying the allografts' fate represent an appealing research domain in transplantation. Its unique characteristics, its promise as accessible biomarker and its therapeutic potential explain the explosion on epigenetic research in medicine. Now it is time for transplantation.

**Acknowledgments**

LH is the recipient of a Ph.D. fellowship of the Research Foundation – Flanders (FWO). BT is the recipient of a postdoctoral fellowship of the FWO.

**Disclosure**

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

## Figure legends

**Figure 1: DNA methylation of a gene promoter represses gene transcription.** This DNA methylation occurs at a cytosine followed by guanine (CpG dinucleotide). Methylation on this palindrome is mostly symmetrical between the top and bottom strand, thereby enabling the propagation of methylation states across cell divisions. Cytosine (C) is methylated (5mC) by DNA methyltransferases (DNMT). Demethylation is achieved by oxidation of the methylgroup to 5-hydroxymethylcytosine (5hmC) through action of the ten-eleven translocation methylcytosine dioxygenase (TET) enzymes and the subsequent removal of the oxidized base by thymine DNA glycosylase (TDG). TTS, transcription start site; DNMT, DNA methyltransferase; TET, ten-eleven translocation methylcytosine dioxygenase enzyme.

**Figure 2: Schematic overview of the perspectives for DNA methylation research in kidney transplantation.**

**Figure 3: DNA methylation events are involved in immune cell development, differentiation and activation.** Hematopoietic stem cells achieve different methylation patterns when differentiating into the myeloerythroid lineage (global hypomethylation) versus lymphoid lineage (global hypermethylation). In various immune cells, differentiation into different cell lines and into activated differentiated cells is accompanied by changes in methylation patterns, relevant

for the function of these cells. In contrast, during the differentiation of effector T and B cells into memory cells the effector phase DNA methylation pattern is maintained, permitting a strong and quick response to antigen rechallenge.

## References


1. Petronis A. Epigenetics as a unifying principle in the aetiology of complex traits and diseases. Nature 2010;465(7299):721-727.
2. Portela A, Esteller M. Epigenetic modifications and human disease. Nature biotechnology 2010;28(10):1057-1068.
3. Schubeler D. Function and information content of DNA methylation. Nature 2015;517(7534):321-326.
4. Bird AP. CpG-rich islands and the function of DNA methylation. Nature 1986;321(6067):209-213.
5. Rauch TA, Wu X, Zhong X, Riggs AD, Pfeifer GP. A human B cell methylome at 100-base pair resolution. Proceedings of the National Academy of Sciences of the United States of America 2009;106(3):671-678.
6. Fazzari MJ, Greally JM. Epigenomics: beyond CpG islands. Nat Rev Genet 2004;5(6):446-455.
7. Hon GC, Rajagopal N, Shen Y, McCleary DF, Yue F, Dang MD et al. Epigenetic memory at embryonic enhancers identified in DNA methylation maps from adult mouse tissues. Nature genetics 2013;45(10):1198-1206.

8. Deaton AM, Webb S, Kerr AR, Illingworth RS, Guy J, Andrews R et al. Cell type-specific DNA methylation at intragenic CpG islands in the immune system. Genome Res 2011;21(7):1074-1086.

9. Illingworth RS, Gruenewald-Schneider U, De Sousa D, Webb S, Merusi C, Kerr ARW et al. Inter-individual variability contrasts with regional homogeneity in the human brain DNA methylome. Nucleic Acids Research 2015.

10. Kikuchi R, Yagi S, Kusuhara H, Imai S, Sugiyama Y, Shiota K. Genome-wide analysis of epigenetic signatures for kidney-specific transporters. Kidney international 2010;78(6):569-577.

11. Marumo T, Yagi S, Kawarazaki W, Nishimoto M, Ayuzawa N, Watanabe A et al. Diabetes Induces Aberrant DNA Methylation in the Proximal Tubules of the Kidney. Journal of the American Society of Nephrology 2015.

12. Cedar H, Bergman Y. Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet 2009;10(5):295-304.

13. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 2009;324(5929):930-935.

14. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 2011;333(6047):1300-1303.

15. Wu H, Zhang Y. Reversing DNA methylation: mechanisms, genomics, and biological functions. Cell 2014;156(1-2):45-68.

16. Xu Y, Wu F, Tan L, Kong L, Xiong L, Deng J et al. Genome-wide regulation of 5hmC, 5mC, and gene expression by Tet1 hydroxylase in mouse embryonic stem cells. Molecular cell 2011;42(4):451-464.

17. Bachman M, Uribe-Lewis S, Yang X, Williams M, Murrell A, Balasubramanian S. 5-Hydroxymethylcytosine is a predominantly stable DNA modification. Nature chemistry 2014;6(12):1049-1055.

18. Metzger RA, Delmonico FL, Feng S, Port FK, Wynn JJ, Merion RM. Expanded criteria donors for kidney transplantation. American Journal of Transplantation 2003;3:114-125.

19. Naesens M. Replicative senescence in kidney aging, renal disease, and renal transplantation. Discovery medicine 2011;11(56):65-75.

20. Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S et al. Genome-wide methylation profiles reveal quantitative views of human aging rates. Molecular cell 2013;49(2):359-367.

21. Jones MJ, Goodman SJ, Kobor MS. DNA methylation and healthy human aging. Aging cell 2015:n/a-n/a.

22. Weidner CI, Ziegler P, Hahn M, Brummendorf TH, Ho AD, Dreger P et al. Epigenetic aging upon allogeneic transplantation: the hematopoietic niche does not affect age-associated DNA methylation. Leukemia 2014.

23. Akintola AD, Crislip ZL, Catania JM, Chen G, Zimmer WE, Burghardt RC et al. Promoter methylation is associated with the age-dependent loss of N-cadherin in the rat kidney. American journal of physiology Renal physiology 2008;294(1):F170-176.

24. Horvath S. DNA methylation age of human tissues and cell types. Genome biology 2013;14(10):R115.

25. Marioni R, Shah S, McRae A, Chen B, Colicino E, Harris S et al. DNA methylation age of blood predicts all-cause mortality in later life. Genome biology 2015;16(1):25.

26. Bröske A-M, Vockentanz L, Kharazi S, Huska MR, Mancini E, Scheller M et al. DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. Nature genetics 2009;41(11):1207-1215.

27. Ji H, Ehrlich LI, Seita J, Murakami P, Doi A, Lindau P et al. Comprehensive methylome map of lineage commitment from haematopoietic progenitors. Nature 2010;467(7313):338-342.

28. Klug M, Heinz S, Gebhard C, Schwarzfischer L, Krause SW, Andreesen R et al. Active DNA demethylation in human postmitotic cells correlates with activating histone modifications, but not transcription levels. Genome biology 2010;11(6):R63.

29. Klug M, Schmidhofer S, Gebhard C, Andreesen R, Rehli M. 5-Hydroxymethylcytosine is an essential intermediate of active DNA demethylation processes in primary human monocytes. Genome biology 2013;14(5):R46.

30. Zhang X, Ulm A, Somineni HK, Oh S, Weirauch MT, Zhang HX et al. DNA methylation dynamics during ex vivo differentiation and maturation of human dendritic cells. Epigenetics & chromatin 2014;7:21.

31. Yang X, Wang X, Liu D, Yu L, Xue B, Shi H. Epigenetic regulation of macrophage polarization by DNA methyltransferase 3b. Molecular endocrinology 2014;28(4):565-574.

32. Lee S, Huen S, Nishio H, Nishio S, Lee HK, Choi BS et al. Distinct macrophage phenotypes contribute to kidney injury and repair. Journal of the American Society of Nephrology : JASN 2011;22(2):317-326.

33. Schmidt IM, Hall IE, Kale S, Lee S, He CH, Lee Y et al. Chitinase-like protein Brp-39/YKL-40 modulates the renal response to ischemic injury and predicts delayed allograft function. Journal of the American Society of Nephrology : JASN 2013;24(2):309-319.

34. Lin SL, Li B, Rao S, Yeo EJ, Hudson TE, Nowlin BT et al. Macrophage Wnt7b is critical for kidney repair and regeneration. Proceedings of the National Academy of Sciences of the United States of America 2010;107(9):4194-4199.

35. Alvarez-Errico D, Vento-Tormo R, Sieweke M, Ballestar E. Epigenetic control of myeloid cell differentiation, identity and function. Nature reviews Immunology 2015;15(1):7-17.

36. Wilson CB, Rowell E, Sekimata M. Epigenetic control of T-helper-cell differentiation. Nature Reviews Immunology 2009;9(2):91-105.

37. Schoenborn JR, Dorschner MO, Sekimata M, Santer DM, Shnyreva M, Fitzpatrick DR et al. Comprehensive epigenetic profiling identifies multiple distal regulatory elements directing transcription of the gene encoding interferon-gamma. Nature immunology 2007;8(7):732-742.

38. Lal G, Zhang N, van der Touw W, Ding Y, Ju W, Bottinger EP et al. Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. The Journal of Immunology 2009;182(1):259-273.

39. Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong J et al. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. European journal of immunology 2007;37(9):2378-2389.

40. Toker A, Engelbert D, Garg G, Polansky JK, Floess S, Miyao T et al. Active demethylation of the Foxp3 locus leads to the generation of stable regulatory T cells within the thymus. Journal of immunology 2013;190(7):3180-3188.

41. Lai AY, Mav D, Shah R, Grimm SA, Phadke D, Hatzi K et al. DNA methylation profiling in human B cells reveals immune regulatory elements and epigenetic plasticity at Alu elements during B-cell activation. Genome Research 2013;23(12):2030-2041.

42. Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. Blood 2009;114(18):3727-3735.

43. Laird PW. The power and the promise of DNA methylation markers. Nat Rev Cancer 2003;3(4):253-266.

44. Bestard O, Cunetti L, Cruzado JM, Lucia M, Valdez R, Olek S et al. Intragraft regulatory T cells in protocol biopsies retain foxp3 demethylation and are protective biomarkers for kidney graft outcome. American journal of

transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2011;11(10):2162-2172.

45. Rodriguez RM, Suarez-Alvarez B, Salvanes R, Muro M, Martinez-Camblor P, Colado E et al. DNA methylation dynamics in blood after hematopoietic cell transplant. PloS one 2013;8(2):e56931.

46. Hodge DR, Peng B, Cherry JC, Hurt EM, Fox SD, Kelley JA et al. Interleukin 6 supports the maintenance of p53 tumor suppressor gene promoter methylation. Cancer research 2005;65(11):4673-4682.

47. Issa J-P. Aging and epigenetic drift: a vicious cycle. The Journal of clinical investigation 2014;124(1):24-29.

48. Ojo AO, Wolfe RA, Held PJ, Port FK, Schmouder RL. Delayed Graft Function: Risk Factors and Implications for Renal Allograft Survival1. Transplantation 1997;63(7):968-974.

49. Salahudeen AK, Haider N, May W. Cold ischemia and the reduced long-term survival of cadaveric renal allografts. Kidney international 2004;65(2):713-718.

50. Yilmaz S, McLaughlin K, Paavonen T, Taskinen E, Monroy M, Aavik E et al. Clinical Predictors of Renal Allograft Histopathology: A Comparative Study of Single-Lesion Histology Versus a Composite, Quantitative Scoring System. Transplantation 2007;83(6):671-676

51. Roodnat J, Mulder P, Van Riemsdijk I, IJzermans J, Van Gelder T, Weimar W. Ischemia times and donor serum creatinine in relation to renal graft failure. Transplantation 2003;75(6):799-804.

52. Franco R, Schoneveld O, Georgakilas AG, Panayiotidis MI. Oxidative stress, DNA methylation and carcinogenesis. Cancer letters 2008;266(1):6-11.

53. Chia N, Wang L, Lu X, Senut MC, Brenner C, Ruden DM. Hypothesis: environmental regulation of 5-hydroxymethylcytosine by oxidative stress. Epigenetics 2011;6(7):853-856.

54. Niu Y, DesMarais TL, Tong Z, Yao Y, Costa M. Oxidative stress alters global histone modification and DNA methylation. Free radical biology & medicine 2015;82:22-28.

55. Huang N, Tan L, Xue Z, Cang J, Wang H. Reduction of DNA hydroxymethylation in the mouse kidney insulted by ischemia reperfusion. Biochem Biophys Res Commun 2012;422(4):697-702.

56. Watson JA, Watson CJ, McCrohan AM, Woodfine K, Tosetto M, McDaid J et al. Generation of an epigenetic signature by chronic hypoxia in prostate cells. Hum Mol Genet 2009;18(19):3594-3604.

57. Watson CJ, Collier P, Tea I, Neary R, Watson JA, Robinson C et al. Hypoxia-induced epigenetic modifications are associated with cardiac tissue fibrosis and the development of a myofibroblast-like phenotype. Hum Mol Genet 2014;23(8):2176-2188.

58. Mariani CJ, Vasanthakumar A, Madzo J, Yesilkanal A, Bhagat T, Yu Y et al. TET1-Mediated Hydroxymethylation Facilitates Hypoxic Gene Induction in Neuroblastoma. Cell reports 2014;7(5):1343-1352.

59. Endres M, Meisel A, Biniszkiewicz D, Namura S, Prass K, Ruscher K et al. DNA methyltransferase contributes to delayed ischemic brain injury. The Journal of Neuroscience 2000;20(9):3175-3181.

60. Robinson CM, Neary R, Levendale A, Watson CJ, Baugh JA. Hypoxia-induced DNA hypermethylation in human pulmonary fibroblasts is associated with Thy-1 promoter methylation and the development of a pro-fibrotic phenotype. Respiratory research 2012;13:74.

61. Pratt JR, Parker MD, Affleck LJ, Corps C, Hostert L, Michalak E et al. Ischemic epigenetics and the transplanted kidney. Transplantation proceedings 2006;38(10):3344-3346.

62. Parker MD, Chambers PA, Lodge JP, Pratt JR. Ischemia- reperfusion injury and its influence on the epigenetic modification of the donor kidney genome. Transplantation 2008;86(12):1818-1823.

63. Zeisberg M, Strutz F, Muller GA. Role of fibroblast activation in inducing interstitial fibrosis. Journal of nephrology 2000;13 Suppl 3:S111-120.

64. Hagood JS. Beyond the genome: epigenetic mechanisms in lung remodeling. Physiology 2014;29(3):177-185.

65. Bian EB, Huang C, Wang H, Wu BM, Zhang L, Lv XW et al. DNA methylation: new therapeutic implications for hepatic fibrosis. Cellular signalling 2013;25(1):355-358.

66. Altorok N, Almeshal N, Wang Y, Kahaleh B. Epigenetics, the holy grail in the pathogenesis of systemic sclerosis. Rheumatology 2014.

67. Kim YS, Kang WS, Kwon JS, Hong MH, Jeong HY, Jeong HC et al. Protective role of 5-azacytidine on myocardial infarction is associated with modulation of macrophage phenotype and inhibition of fibrosis. Journal of cellular and molecular medicine 2014;18(6):1018-1027.

68. Bechtel W, McGoohan S, Zeisberg EM, Muller GA, Kalbacher H, Salant DJ et al. Methylation determines fibroblast activation and fibrogenesis in the kidney. Nature medicine 2010;16(5):544-550.

69. Tampe B, Tampe D, Muller CA, Sugimoto H, LeBleu V, Xu X et al. Tet3-mediated hydroxymethylation of epigenetically silenced genes contributes to bone morphogenic protein 7-induced reversal of kidney fibrosis. Journal of the American Society of Nephrology : JASN 2014;25(5):905-912.

70. Bell CG, Teschendorff AE, Rakyan VK, Maxwell AP, Beck S, Savage DA. Genome-wide DNA methylation analysis for diabetic nephropathy in type 1 diabetes mellitus. BMC medical genomics 2010;3(1):33.

71. Zawada AM, Rogacev KS, Hummel B, Grün OS, Friedrich A, Rotter B et al. SuperTAG methylation-specific digital karyotyping (SMSDK) reveals uremia induced epigenetic dysregulation of atherosclerosis-related genes. Circulation: Cardiovascular Genetics 2012:CIRCGENETICS. 112.963207.

72. Ko YA, Mohtat D, Suzuki M, Park AS, Izquierdo MC, Han SY et al. Cytosine methylation changes in enhancer regions of core pro-fibrotic genes characterize kidney fibrosis development. Genome biology 2013;14(10):R108.

73. Wing MR, Devaney JM, Joffe MM, Xie D, Feldman HI, Dominic EA et al. DNA methylation profile associated with rapid decline in kidney function: findings

from the CRIC study. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 2014;29(4):864-872.

74. Stenvinkel P, Karimi M, Johansson S, Axelsson J, Suliman M, Lindholm B et al. Impact of inflammation on epigenetic DNA methylation – a novel risk factor for cardiovascular disease? Journal of Internal Medicine 2007;261(5):488-499.

75. Sapienza C, Lee J, Powell J, Erinle O, Yafai F, Reichert J et al. DNA methylation profiling identifies epigenetic differences between diabetes patients with ESRD and diabetes patients without nephropathy. Epigenetics 2011;6(1):20-28.

76. Dwivedi RS, Herman JG, McCaffrey TA, Raj DS. Beyond genetics: epigenetic code in chronic kidney disease. Kidney international 2011;79(1):23-32.

77. Sun CY, Chang SC, Wu MS. Suppression of Klotho expression by protein-bound uremic toxins is associated with increased DNA methyltransferase expression and DNA hypermethylation. Kidney international 2012;81(7):640-650.

78. Chen J, Zhang X, Zhang H, Lin J, Zhang C, Wu Q et al. Elevated Klotho promoter methylation is associated with severity of chronic kidney disease. PloS one 2013;8(11):e79856.

79. Gielis EM, Ledeganck KJ, De Winter BY, Del Favero J, Bosmans JL, Claas FHJ et al. Cell-Free DNA: An Upcoming Biomarker in Transplantation. American Journal of Transplantation 2015:n/a-n/a.

80. Gertz J, Varley KE, Reddy TE, Bowling KM, Pauli F, Parker SL et al. Analysis of DNA methylation in a three-generation family reveals widespread genetic influence on epigenetic regulation. PLoS genetics 2011;7(8):e1002228.

81. Heyn H, Esteller M. DNA methylation profiling in the clinic: applications and challenges. Nat Rev Genet 2012;13(10):679-692.

82. Moisiadis VG, Matthews SG. Glucocorticoids and fetal programming part 2: Mechanisms. Nat Rev Endocrinol 2014;10(7):403-411.

83. Falahi F, Sgro A, Blancafort P. Epigenome engineering in cancer: fairytale or a realistic path to the clinic? Front Oncol 2015;5:22.

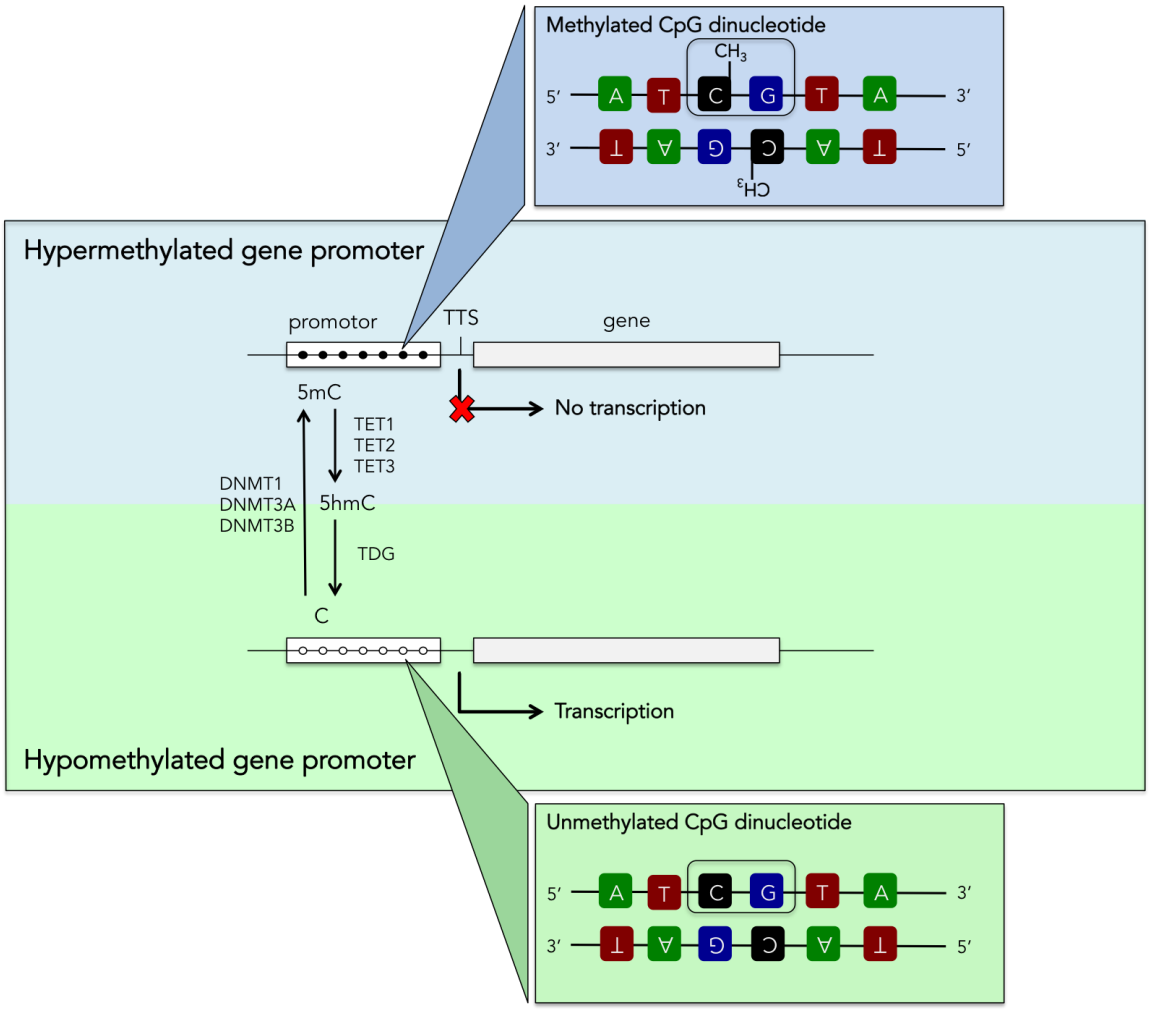


***Figure 1: DNA methylation of a gene promoter represses gene transcription.*** *This DNA methylation occurs at a cytosine followed by guanine (CpG dinucleotide).* Methylation on this palindrome is mostly symmetrical between the top and bottom strand, thereby enabling the propagation of methylation states across cell divisions. *Cytosine (C) is methylated (5mC) by DNA methyltransferases (DNMT). Demethylation is achieved by oxidation of the methylgroup to 5-hydroxymethylcytosine (5hmC) through action of the ten-eleven translocation methylcytosine dioxygenase (TET) enzymes and the subsequent removal of the oxidized base by thymine DNA glycosylase (TDG). TTS,*

*transcription start site; DNMT, DNA methyltransferase; TET, ten-eleven translocation methylcytosine dioxygenase enzyme.*

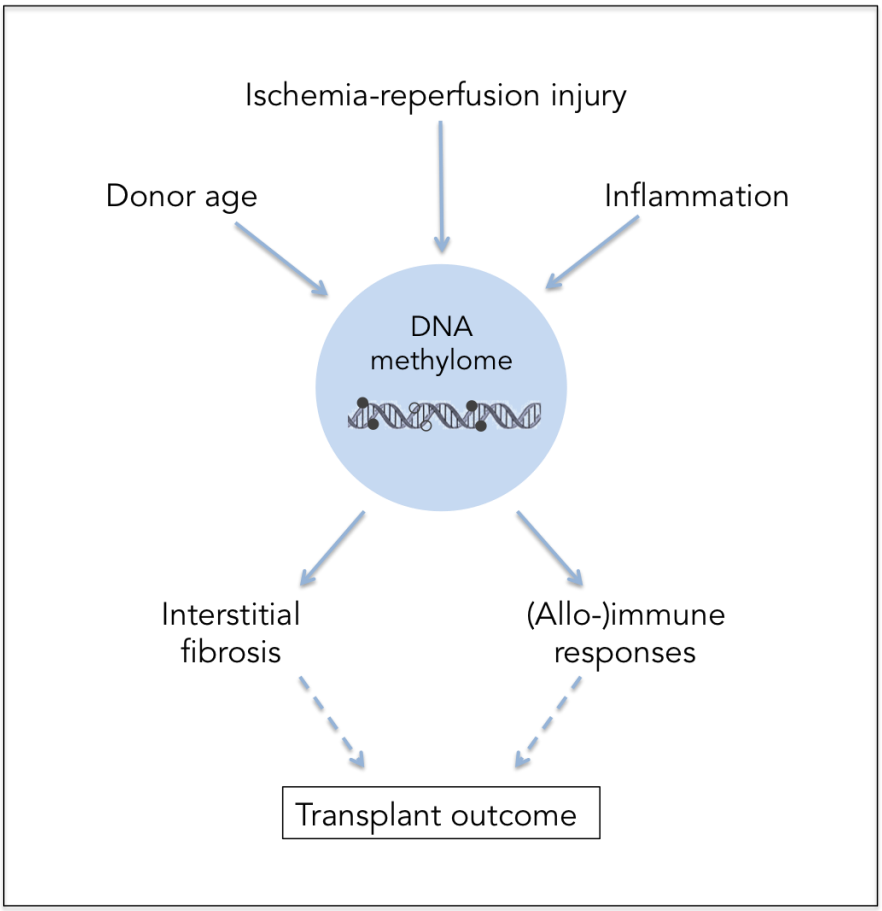


**Figure 2: Schematic overview of the perspectives for DNA methylation research in kidney transplantation**

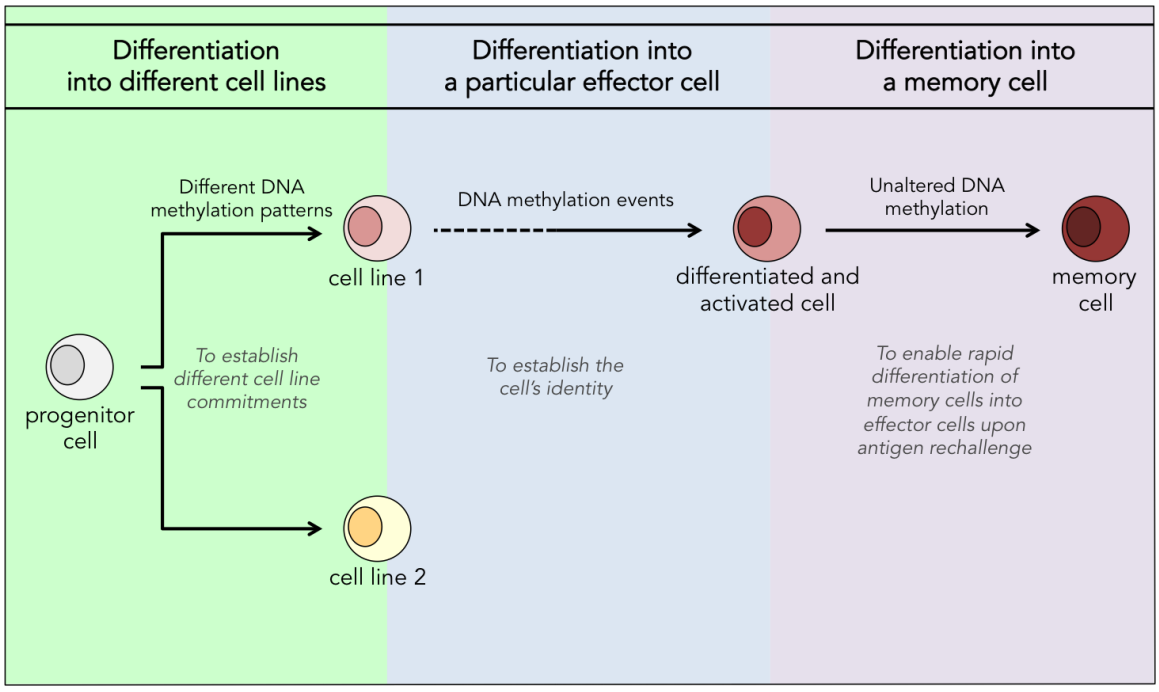


***Figure 3. DNA methylation events are involved in immune cell development, differentiation and activation.*** *Hematopoietic stem cells achieve different methylation patterns when differentiating into the myeloerythroid lineage (global hypomethylation) versus lymphoid lineage (global hypermethylation). In various immune cells, differentiation into different cell lines and into activated differentiated cells is accompanied by changes in methylation patterns, relevant for the function of these cells. In contrast, during the differentiation of effector T and B cells into memory cells the effector phase DNA methylation pattern is maintained, permitting a strong and quick response to antigen rechallenge.*